# Synergistic effect of carbon nuclei and polyaromatic hydrocarbons on respiratory and immune responses



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## ABSTRACT

**Background:** Particulate matter with aerodynamic diameter ≤2.5 μm ($PM_{2.5}$) is generally composed of carbon nuclei associated with various organic carbons, metals, ions and biological materials. Among these components, polyaromatic hydrocarbons (PAHs) such as benzo(a)pyrene (BaP) and quinones have detrimental effects on airway epithelial cells and immunodisrupting effects, which leads to the exacerbation of respiratory allergies. The effects of PAHs and the carbon nuclei, separately as well as in combination, remain to be established.

**Objective:** We investigated the effects of BaP, 9,10-phenanthroquinone (9,10-PQ) and 1,2-napthoquinone (1,2-NQ) and their combined effects with heated diesel exhaust particle (H-DEP) as carbon nuclei of typical $PM_{2.5}$.

**Methods:** We exposed human airway epithelial cells (BEAS-2B), murine bone marrow-derived antigen-presenting cells (APCs), and murine splenocytes to BaP, 9,10-PQ, or 1,2-NQ in the presence and absence of H-DEP. Several important inflammatory cytokines and cell surface molecules were measured.

**Results:** PAHs alone did not have apparent cytotoxic effects on BEAS-2B, whereas combined exposure with H-DEP induced noticeable detrimental effects which mainly reflected the action of H-DEP itself. BaP increased CD86 expression as an APC surface molecule regardless of the presence or absence of H-DEP. None of the BaP, 9,10-PQ or 1,2-NQ exposure alone or their combined exposure with H-DEP resulted in any significant activation of splenocytes.

**Conclusions:** These results suggest that PAHs and carbon nuclei show additive effects, and that BaP with the carbon nuclei may contribute to exacerbations of allergic respiratory diseases including asthma by $PM_{2.5}$, especially via APC activation.

## Introduction

It is clear from decades-long studies (Ring et al, 2001) that the exacerbation of respiratory allergies is associated with particulate matter with aerodynamic diameters ≤10 μm ($PM_{10}$) and that with aerodynamic diameters ≤2.5 μm ($PM_{2.5}$). Particulate matters are usually released into the air from sources such as the burning of coal and wood, the combustion of petrol and diesel, and a variety of industrial processes. The aforementioned emissions included polyaromatic hydrocarbons (PAHs) associated with carbon particle and metal (Tobiszewski and Namiesnik, 2012).

PAHs, including benzo[a]pyrene (BaP) and quinones, are among the most prominent compounds found in $PM_{2.5}$. Diesel exhaust particles (DEPs) contains PAHs that cause the exacerbation of allergic respiratory diseases including asthma. Several studies have shown BaP and quinones also have a strong association with the increased incidents of allergic airway disorder (Kadkhoda et al, 2004, Inoue et al, 2007, Hiyoshi et al, 2005, Kumagai et al, 2007). In addition, quinones are important agents that affect health, and they are considered responsible for modulating the metabolic profile and DNA damage in respiratory cells (Gurbani

et al, 2013). Several studies established that *ortho*-quinone has a higher cytotoxic effect than *para*-quinone in airway epithelial cells (Koike et al, 2014) which may indicate that *ortho*-quinone contributes to exacerbations of airway inflammation. 9, 10-phenanthroquinone (9,10-PQ) and 1, 2-naphthoquinone (1,2-NQ) are both examples of *ortho*-quinone (Fig. 1).

Regarding the health effects of $PM_{2.5}$, the synergy of organic components and the nuclei themselves has been reported (Yanagisawa et al, 2006). It is thus necessary to determine the effects of PAHs alone and their co-exposure with a carbon nucleus to understand the mechanisms by which $PM_{2.5}$ leads to the exacerbation of allergic diseases. However, the combined effects of BaP or quinones and carbon nuclei have not been investigated.

Our objective in the present study was to observe the effects of BaP, 9,10-PQ and 1,2-NQ and their combined effects with the carbon nuclei on respiratory cells as well as lymphocytes and antigen-presenting cells (APCs), to understand the factors responsible for the vulnerability of respiratory allergy to PM2.5.

## Methods

### *Cell preparation*

The experiment was performed on human airway epithelial cells (BEAS-2B) and immune cells including splenocytes and bone marrow-derived cells (APCs). Ten-week-old NC/NgaTendCrlj male mice (Chares River Japan, Osaka, Japan) were sacrificed by cervical dislocation and

exsanguinated by cutting the abdominal aorta and vein. Splenocytes and APCs were extracted from each mouse. All animal study procedures were approved by the Animal Research Committee at Kyoto University.

*Airway epithelial cells*

The BEAS-2B cell line, which is derived from human bronchial epithelial cells transformed by an adenovirus (12-SV40 hybrid virus) was purchased from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK).

To initiate the cell culture, the vial containing the cells was taken out from liquid nitrogen and added into serum-free LHC-9 medium (Life Technologies, Carlsbad, CA, USA). The subculture was maintained in LHC-9 medium in an incubator in a 5% $CO_2$ atmosphere at 37°C. For particular experiments, cells were seeded in 96- and 12-well collagen I-coated plates and incubated for 72 hr to reach semi-confluence in LHC-9 medium with the same conditions as those used for the subculture.

*Bone marrow isolation*

Cells were isolated from the femur bone of two legs of each mouse. After the surrounding muscle tissue was removed, the bones were left in 70% ethanol for 3 min and washed with RPMI medium. Both ends of the bones were cut and the marrow was flushed with RPMI

medium using a syringe with a 25G needle. The marrow suspension was passed through a sterile nylon mesh to remove small pieces of bone and debris, and the red blood cells were lysed with hemolytic reagent (ammonium chloride based). The cells were centrifuged at 400*g* for 5 min at 20°C.

After aspiration of the media, the cells were resuspended in culture medium R10, which was GIBCO RPMI 1640 medium (Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; MP Biomedicals, Eschwege, Germany), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO), and 50 µM 2-mercaptoethanol (Invitrogen).

*Differentiation of APCs*

APCs were differentiated using a modification of the protocol described by Lutz et al, 1999. In brief, bone marrow cells ($4\times10^5$ /mL) were cultured in R10 medium containing 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF). On day 3, the same volume of the medium containing 20 ng/mL GM-CSF was added to the culture. On day 6, half the culture medium was replaced with fresh medium. On day 8, non-adherent and loosely adherent cells were collected by gentle pipetting. The differentiated APCs were centrifuged at 400*g* for 5 min at 20°C and then resuspended in fresh medium.

The numbers of viable cells were determined by the trypan blue exclusion method. The

final concentration of the cell suspension and exposure medium was appropriately calculated to achieve the final concentration as $1\times10^6$ cells/ml.

*Splenocyte isolation*

The spleen was removed from the mice, crushed and pushed through a 200-mesh stainless steel sheet. The cells were suspended in RPMI-1640 media and centrifuged at 400*g* for 5 min at 20°C. After being treated with hemolytic reagent, the cell suspension was centrifuged one more time. The numbers of viable cells were determined by the trypan blue exclusion method. The final concentrations of the cell suspension and exposure medium were appropriately calculated to achieve the final concentration of $1\times10^6$ cells/ml.

*Exposure of airway epithelial cells and immune cells*

We examined BaP (purchased from Sigma-aldrich), 9,10-PQ (purchased from Sigma aldrich), and 1,2-NQ (purchased fom Tokyo chemical Industry) as exposure reagents. To understand the synergistic effects of carbon nuclei and PAHs in DEP as representative of $PM_{2.5}$, we used heated-DEP (H-DEP) where PAHs were excluded by heat at 360°C for 30 min. We characterized the H-DEP particles and measured various components including several PAHs consisting of four- five– and six-membered rings. The particle did not have any PAHs associated with it. Heating at 360°C caused complete evaporation of PAH. However, metals

and ions such as, Ca, Na, Zn, Fe, Mn, As and $SO_4^{2-}$ had been detected. Cells were exposed to BaP (0, 0.1, 1.10 μM) or 9,10-PQ or 1,2-NQ (0, 0.01, 0.1, 1 μM for both) in the presence or absence of H-DEP (50 μg/mL) for 24 or 72 h. The results of our pre-experiments using quinone showed that doses higher than the aforementioned doses are highly cytotoxic and thus not appropriate for the immune study.

To measure the cell viability and the interleukin (IL)-6 and IL-8 releases from airway epithelial cells, we performed a water-soluble tetrazolium salt-1 (WST-1) assay and enzyme-linked immunosorbent assay (ELISA), respectively. We also measured the cell viability and the expression of cell surface molecules of APCs (DEC205, a dendritic cell marker; CD86, an APC marker) by performing a WST-1 assay and fluorescence-activated cell sorter (FACS) analysis, respectively. The cell viability, cell proliferation and the expression of cell surface molecules of splenocytes (T-cell receptor [TCR], a T-cell marker; CD19, a B-cell marker) were measured by WST-1 assay, 5-bromo-2'-deoxyuridine (BrdU) ELISA, and FACS analysis, respectively.

*Cell viability*

Cell viability was assessed by WST-1 assay. The BEAS-2B cell suspension of $7.5\times10^4$ cells/mL was seeded at 70 μL/well in collagen I-coated 96-well plates and cultured for 3 days. On day 3, the medium was discarded and exposed to the sample solution at an equal concentration by 70 μL/well. After 21 hr of exposure, the WST-1 reagent was added. A WST-1 reagent amount

should be 1/10th of the sample volume; for this experiment, it was thus 7 μL. After 3 hr under WST-1 exposure, the absorbance of the plate was measured at 450 nm using a microplate reader (reference wavelength 630 nm).

Similarly, APCs and splenocytes were cultured for 23 hr 30 min and 20 hr, respectively, and then treated with a 1/10th amount of sample volume of WST-1 reagent for 30 min for the APCs and 4 hr for the splenocytes. Thereafter, the absorbance of the plate was measured at 450 nm using a microplate reader (reference wavelength 630 nm).

*Cell proliferation assay*

To estimate the splenocytes' proliferation, we performed a BrdU analysis. According to the instructions of the BrdU assay kit (Roche Lifescience). The cells were exposed for 3 days and subjected to absorbance measurement at wavelength 450 nm with the reference wavelength 630 nm. BrdU was added at 20 hr prior to the measurement.

*Quantification of cytokines in culture supernatant by ELISA*

After exposure to a PAH and the combination of PAH + H-DEP, BEAS-2B cells were cultured for 24 hr and centrifuged at 300*g* for 5 min, and the supernatant was collected. The supernatant was stored at −80°C. The levels of IL-6 and IL-8 released from BEAS-2B cells were measured by quantikine ELISA kits (IL-6 and IL-8 from Thermo Scientific). The IL-10 level of the APCs

was also determined by a quantikine ELISA kit (Thermo Scientific).

*FACS analysis*

The cell surface molecules were measured by a FACS analysis. The following monoclonal antibodies were used to react with specific antigens: Mouse BD Fc Block™ purified anti-mouse CD16/CD32 (Becton Dickinson), DEC205 (NLDC-145, PE-conjugated, BioLegend, San Diego, CA), Rat IgG2a, κ Isotype Control (RTK2758, PE-conjugated, BioLegend), CD86 (GL-1, PE-conjugated, Becton Dickinson), Rat IgG2a, κ Isotype Control (R35-95, PE-conjugated, Becton Dickinson), Hamster Anti-Mouse TCR-β Chain (H57-597, FITC-conjugated, Becton Dickinson), Hamster IgG2, λ1 Isotype Control (Ha4/8, FITC-conjugated, Becton Dickinson), Rat Anti-Mouse CD19 (1D3, PE-conjugated, Becton Dickinson), and Rat IgG2a, κ Isotype Control (R35-95, PE-conjugated, Becton Dickinson). To determine the antigen-positive zones in the FACS histogram, we compared stained samples with un-stained and isotype controls (Fig. 2).

After exposure for 24 hr, the immune cells were collected and resuspended in 50 μL phosphate-buffered saline (PBS) with 0.3% bovine serum albumin and 0.05% sodium azide (Wako Pure Chemical Industries, Osaka, Japan). Depending on the objective marker, the appropriate antibody was added into it and maintained for 45 min at 4°C before a wash with FACS buffer. The cell surface molecules were measured with a FACS Calibur (Becton

Dickinson). For the analysis, the percentage data of positive cells with a particular marker per 10,000 cells was determined.

*Statistical analysis*

The experiments for cytotoxicity, the release of cytokines, and the cell surface molecule expression were performed with multiple samples (n=3–4). The average values ± standard error of the mean (SEM) were calculated for all statistical analyses. Intergroup differences were examined by Tukey's multiple comparison tests. A p-value <0.05 was considered significant.

## Results

*Effects on respiratory responses*

Exposure to BaP alone at higher doses increased the cell viability of the airway epithelial cells. Neither of the two quinones alone changed cell viability. H-DEP alone lowered cell viability. BaP + H-DEP (1 µM and 10 µM) exposure resulted in high viability (referred with '+' sign in Fig. 3A) compared to the control. Combined exposure to 9,10-PQ + H-DEP and 1,2-NQ + H-DEP at some doses decreased the cell viability compared to single-exposure (Fig. 3B,C).

With BaP exposure, the IL-6 expression did not show any significant difference (Fig. 4A). The IL-8 expression did not change significantly with BaP exposure either (Fig. 4D). The quinones' single exposure did not result in any changes in IL-6 or IL-8 expression. The PAHs

with or without H-DEP also did not result in any noticeable pro-inflammatory changes (Fig. 4).

*Effects on immune responses*

Effects on APCs

BaP single exposure decreased the viability of APCs (Fig. 5A). The quinones alone had a feeble decreasing effect on cell viability. H-DEP alone did not change cell viability as well. BaP + H-DEP caused low cell viability compared to the control. 9,10-PQ + H-DEP and 1,2-NQ + H-DEP exposure both did not show any change in viability compared to control except highest dose of the later, which lowered the viability (Fig. 5B,C).

BaP single exposure showed high CD86 expression (Fig. 6A) while quinones did not have any effect on any dose. H-DEP alone also did not show any noticeable effect. BaP+H-DEP had an effect on the elevation of CD86 expression as compared to control. But combined exposure to the quinones and H-DEP did not have any significant effect (Fig. 6B and 6C). On the other hand, PAHs with or without H-DEP did not cause any change in the expression of DEC205 (Fig. 7). IL-10 expression for all of the PAHs and their combined exposure showed the result under detection level.

Effects on splenocytes

BaP single exposure decreased cell viability significantly after 24 hr of exposure (Fig. 8A).

Both of the quinones did not show any difference in single exposures except at 1 µM dose of 9,10-PQ that showed significantly decreased viability (Fig. 8B,8C). H-DEP alone tended to decrease cell viability. BaP + H-DEP showed the significant detrimental effect on cell viability as compared to control. 9,10-PQ + H-DEP and 1,2-NQ + H-DEP tended to decrease cell viability as compared to control.

When we checked the proliferation of splenocytes after 72 hr of exposure, we observed that BaP single exposure (1 µM and 10 µM) decreased proliferation (Fig. 8D). Neither 9,10-PQ nor 1,2-NQ single exposure had a significant effect. H-DEP alone as well as BaP + H-DEP, 9,10-PQ + H-DEP and 1,2-NQ + H-DEP had declining effects on cell proliferation compared to single exposure (Fig. 8).

Figure 9 summarizes the following results. BaP single exposure did not cause changes in TCR expression. However, 9,10-PQ single exposure at 1 µM resulted in a significantly high expression of TCR (Fig. 9B). 1,2-NQ single exposure did not have any effect. H-DEP alone did not show any change. 9,10-PQ + H-DEP at the 1 µM dose tended to have an increasing effect compared to the control.

BaP exposure did not change CD19 expression. The single-exposure of quinones also did not result in any change in CD19 expression. H-DEP alone also did not have any noticeable effect. BaP + H-DEP did not have any effect, but 9,10-PQ + H-DEP at 1 µM decreased the CD19 expression (Fig. 9E). 1,2-NQ + H-DEP exposure did not affect the CD19 expression (Fig.

248 9F).

249

250 **Discussion**

251 Here we observed that the PAHs alone did not have apparent cytotoxic effects on airway
252 epithelial cells, whereas the combined exposure of 9,10-PQ or 1,2-NQ with H-DEP induced a
253 noticeable detrimental effect, which mainly reflected the action of H-DEP. No evidence was
254 obtained indicating that considered PAHs or combined exposures have any pro-inflammatory
255 effect on airway epithelial cells. BaP had a significant impact on the immune response by
256 stimulating APCs via CD86 to enhance co-stimulatory molecules' expression.

257 H-DEP exposure did not result in any noticeable increase of CD86, but BaP and H-DEP
258 showed synergistic effects (under conditions in which BaP or H-DEP sometimes affected cell
259 viability). 9,10-PQ and 1,2-NQ and their combined exposure with H-DEP did not show any
260 significant activation of APCs. H-DEP inhibited splenocyte proliferation. No significant
261 activation of splenocytes was caused by BaP, 9,10-PQ or 1,2-NQ single-exposure or by
262 combinations of them with H-DEP.

263 In 2008, Goulaovic et al. used monocyte leukemia cells to observe the pro-inflammatory
264 cytokine reaction of microphages under carbon black + PAHs. They found that BaP has pro-
265 inflammatory effects whereas ultra-fine particles (14 nm dia.) have immunotoxic effects. They
266 also noted that BaP with ultrafine particles partially amplified the immunotoxic effect. In a very

similar way, we conducted our present experiment to investigate the effects of the well-studied PAH, such as BaP compared to the effects of quinones 9,10-PQ and 1,2-NQ, which are not yet widely studied but a potential contributor of respiratory allergy, in the presence and absence H-DEP. In contrast to Goulaouic et al.'s study, our focus was the scope of respiratory allergy, i.e., airway epithelial cells and immune cells of atopic prone NC/Nga mouse. Based on the previous reports and the present status of our knowledge, the present study is the first to elucidate a synergistic effect in respiratory cells.

Airway epithelial cells act as the first line of defense against xenobiotics in the innate immune system. They are capable of secreting cytokines such as IL-6 and IL-8, and thereafter via a trans-signaling mode, they are critically involved in the pathogenesis of inflammatory response. In the present study, we observed that the single exposures of PAHs did not have a significant effect on cell viability, except for BaP, which significantly increased cell viability ($p$<0.01) at 1 and 10 μM doses in airway epithelial cells (BEAS-2B) cells. We also observed that most of the combined exposures with H-DEP decreased cell viability compared to the PAHs alone. It has been reported that carbon nano and micron size particles decreased cell viability size and dose-dependently (Sahu et al, 2014), and the combined-effect cytotoxicity in the present study might thus be the result of mainly the carbon nuclei.

Experimental studies with DEP or ambient $PM_{2.5}$ indicated that pro-inflammatory responses such as IL-6 and IL-8 release occur in a irway epithelial cells (Streerenberg, 1998).

Other investigators demonstrated the inhibition of IL-8, but an elevation in IL-6 expression (Fuentes et al, 2010, Rodriguez-cotto, 2014). Depending on the components of exposure, the IL-6 and IL-8 expressions change, because the pathophysiological mechanisms are different. To understand how our chosen PAHs influences the pro-inflammatory effect, we analyzed the IL-6 and IL-8 expressions. Although we did not observe significant changes in IL-6 expression due to any of the PAHs, the steady increase of IL-6 with a single exposure to BaP should not be overlooked. Hu et al., 2016, recently reported that the aryl hydrocarbon receptor (AhR) is a signaling pathway that is a probable cause of the increase of IL-6. It is already established that BaP is an AhR ligand (Beamer and Shepherd, 2013). According to them it seems likely that after entering the body BaP goes through a cascade of intra-cellular reaction with AhR and eventually reaches a range of endpoints including aberrant cytokine secretion.

In the present study, none of the PAHs apparently caused inflammatory responses, but BaP might have triggered IL-6 release via some signal transductions as the IL-6 secretion was greater than that of the control. On the other hand, IL-8 did not change significantly but their dose dependent result should not be overlooked. The low dose of 9,10-PQ somewhat elevated the IL-8 secretion but the 1 µM dose downregulated the IL-8 secretion but the change is not statistically significant. The 9,10-PQ+H-DEP exposure resulted in slight IL-8 elevation, whereas Bap + H-DEP showed no such variation. Wang et al. 2012, indicated that IL-8 is regulated by the phosphorylation of Erk1/2, whereas the phosphorylation of p38 inhibits it. It

was also reported that PAHs including BaP induced ERK1/2 and p38 kinases (Andrysik, 2016). Though it has not been confirmed that PAHs trigger the same biological pathway of IL-8 upregulation or downregulation, it is possible that 9,10-PQ at 1 μM and combined-exposure quinones with H-DEP may sometimes follow the same pathway. Alfaro-Moreno et al.2009 reported a sharp IL-8 decrease and an IL-6 increase in airway epithelial cells exposed to particulate matter, which somewhat resembles our results with BaP single-exposure and 9,10-PQ + H-DEP (except at 0.01 μM).

Respiratory allergies such as asthma are mediated by Th2 responses, in which upon their recognition of an invasive allergen, APCs process the allergen into small peptides for presentation on major histocompatibility complex (MHC) and migrate to secondary lymphoid tissue. Subsequently, the matured and activated APC-allergen combination attaches to specific TCRs on T cells and induces the proliferation of lymphocytes (T and B cells). On the other hand, APCs are known to produce IL-10, which is essential for a Th2 response.

In APCs, the expression of CD86 was significant after BaP single and combined exposures in our study. Yanagisawa et al. 2016 obtained similar results in their study, in which the numbers of $CD86^{+}$ and MHC class $II^{+}$ cells feebly increased with BaP exposure of Ovalbumin sensitized mediastinal lymph node (MLN) cells. The MHC class II and co-stimulatory molecule CD86 is essential for antigen presentation. According to Li et al.,2016 CD86 is a key factor of Th1/Th2 cytokine regulation. It has been noted that APCs express co-

stimulatory molecules that include CD86, which is responsible for providing the second signal for the optimal induction of T-cell activation, division, and differentiation. It is evident that the patients with allergic asthma has high CD86 in their Bronchoalveolar lavage (BAL) fluid and it is concluded that CD86 has a role to play in allergen-induced inflammatory process in asthmatic pathway (Liang et al, 2006).

In the present study, BaP rather than H-DEP exaggerated immune responses through the activation of APCs. BaP in the presence and the absence of heated-Asian sand dust particles increased the CD86 expression on APCs (Honda et al., in press). This result is in accord with our novel findings regarding H-DEP; that is, (1) BaP adhered to the particles has greater effects than the particles themselves and (2) BaP and particles have additive/synergistic effects on CD86 expression, which can be expected to be important in the initiation of Th2 responses, which eventually trigger respiratory allergies such as asthma.

According to the findings of Kadkhoda et al., 2005, IL-10 from APCs is essential for the Th2 response, and the IL-10 expression increased under BaP exposure. In contrast, Hwang et al.2007 reported that IL-10 production was impaired under BaP exposure. Our present investigation did not reveal either of those variations (data not shown). IL-10 secretion is an antigen-specific reaction (Yanaba et al., 2008). We therefore suspect that the PAHs examined herein without antigenicity did not trigger the IL-10 molecule.

DEC205/CD205 molecule, which is known for mediating the capture and internalization of ligands for processing and presentation by APCs, was not affected by our chosen PAHs alone or by their combined exposure with H-DEP. On the basis of the present results, BaP and the two quinones could not affect the antigen uptake.

Splenocytes' viability and proliferation seem to be linked, as the combined exposure to all of the PAHs studied here inhibited both the viability and the proliferation of the splenocytes. TCR is an appropriate marker of T cells because it interacts with the MHC antigen peptide complex on APCs, and CD28 molecules expressed on the surface of T cells interact with CD80 and CD86 ligands expressed on the surface of APCs (Li et al, 2016). Considering this costimulatory pathway, we measured TCR on splenocytes. Although the cell viability was significantly low with the 9,10-PQ exposure at the highest dose (1 μM), the TCR expression was highest in that case. These results may be interpreted as indicating that under those doses, other kind of cell types in splenocytes had degenerated except T cells, and thus the number of TCR-positive cells seemed to increase. 1,2-NQ + H-DEP exposure resulted in a slight negative for TCR expression, but the difference was not significant. CD19 deficiency is responsible for a worse inflammatory response (Lordan et al, 2000). It is thus reasonable to infer that 9,10-PQ has the potential to trigger inflammation at a higher dose with H-DEP by inhibiting CD19 expression. Comparing the TCR and CD19 percentages, the numbers of T cells were increased whereas those of B cells were decreased at the high dose of 9,10-PQ, both in single and

combined exposure. This may explain why the B cells had higher sensitivity against 9,10-PQ compared to the T cells.

In conclusion, our results established that the synergistic exposure to PAHs and the carbon nuclei is notably detrimental compared to a single exposure, at least under *in vitro* conditions. The 9,10-PQ or 1,2-NQ alone may not be the principle contributing factor to the airway inflammation. However, in combined exposure with carbon nuclei, they impart observable detrimental effects. BaP can contribute to the development and/or exacerbation of respiratory allergies such as asthma, especially to the immune system via APC activation. As in ambient air, $PM_{2.5}$ is always associated with PAHs and other material, and we propose that a much more realistic approach to study the effects of particulate matter is to investigate exposure to their combinations.

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**Figure legends**

**Fig. 1.** Structures of benzo(a)pyrene, 9,10-phenanthroquinone (9,10-PQ) and 1,2-nephthoquinone (1,2-NQ).

**Fig. 2.** Representative data for unstained (black), isotype control (gray) and CD86-positive cells (filled gray). The histogram shows the percentage of CD86-positive cells with subset bars.

**Fig. 3.** Cell viability of BEAS-2B cells exposed to (**A**) BaP, (**B**) 9,10-PQ and (**C**) 1,2-NQ. *p<0.05 vs. corresponding control. #p<0.05 vs. same concentration.

**Fig. 4.** Levels of IL-6 and IL-8 produced by BEAS-2B cells exposed to (**A&D**) BaP, (**B&E**) 9,10-PQ and (**C&F**) 1,2-NQ.

**Fig. 5.** Cell viability of APC was performed by WST-1 Assay to understand the effect of (**A**) BaP, (**B**) 9,10-PQ and (**C**) 1,2-NQ in APC. *p<0.05 vs. corresponding control.

**Fig. 6.** CD86 positive cells in APC during exposure of (**A**) BaP, (**B**) 9,10-PQ and (**C**) 1,2-NQ in APC, measured by FACS. *p<0.05 vs. corresponding control.

**Fig 7.** DEC205 positive cells in APC during exposure of (**A**) BaP, (**B**) 9,10-PQ and (**C**) 1,2-NQ in APC, measured by FACS.

518 **Fig. 8.** Cell viability of splenocyte and cell proliferation during exposure of (**A&D**) BaP,
519 (**B&E**) 9,10-PQ and (**C&F**) 1,2-NQ. *p<0.05 vs. corresponding control. #p<0.05 vs. same
520 concentration.

521

522 **Fig. 9.** TCR and CD19 positive cells in splenocytes during exposure of (**A&D**) BaP, **(B&E**)
523 9,10-PQ and (**C&F**) 1,2-NQ in BEAS-2B cell line was measured by FACS. *p<0.05 vs.
524 corresponding control.

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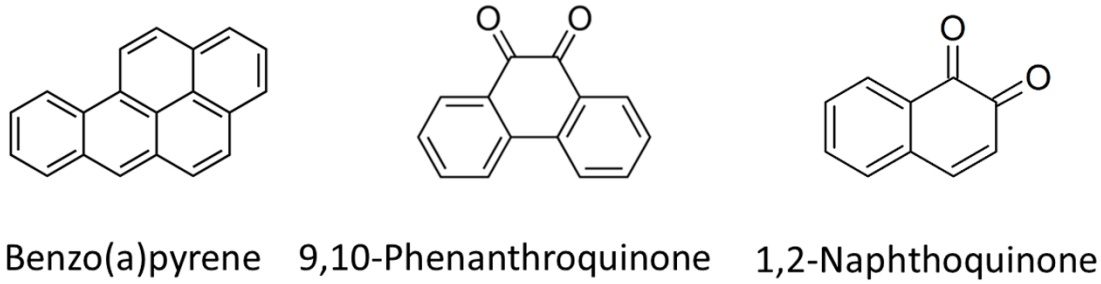
533 Fig.1

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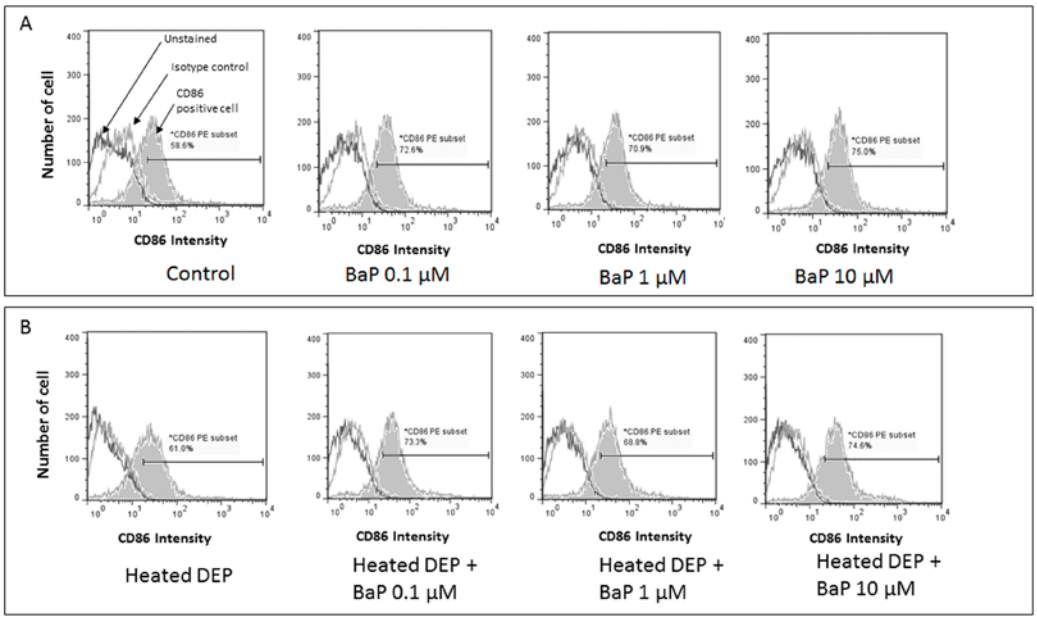


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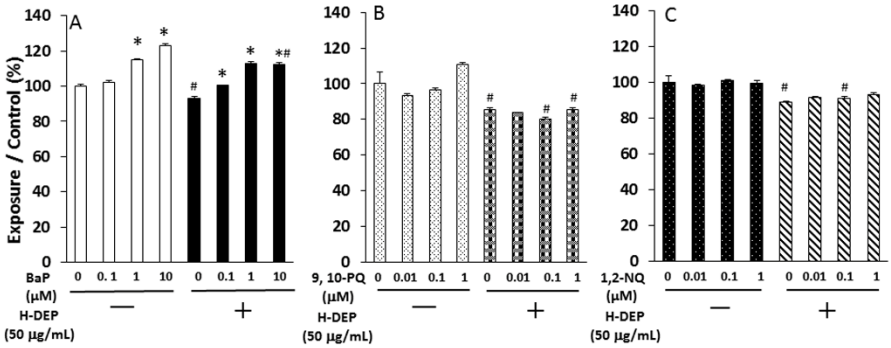


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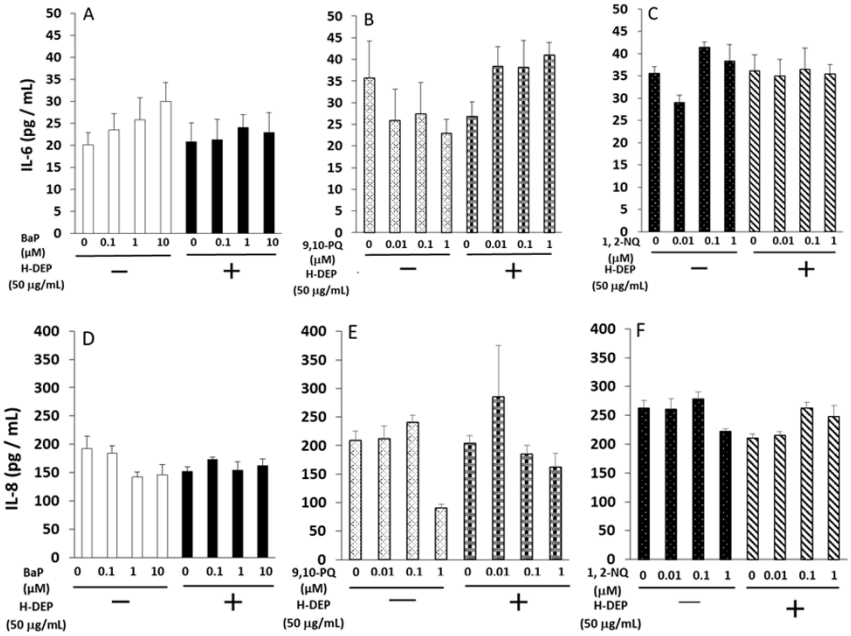


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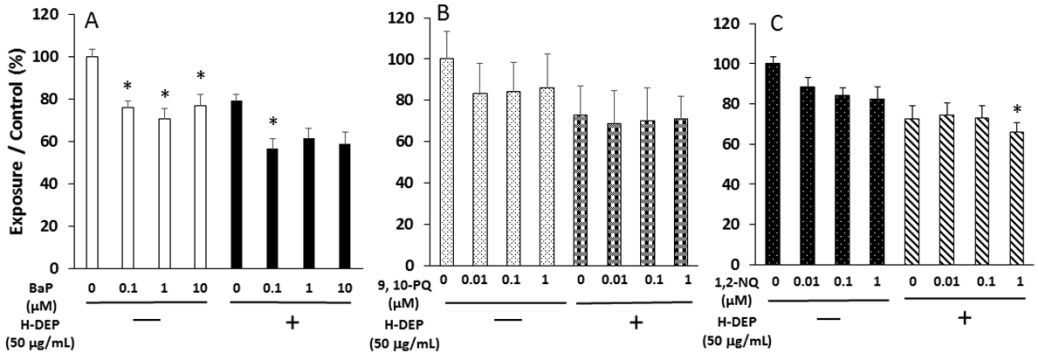


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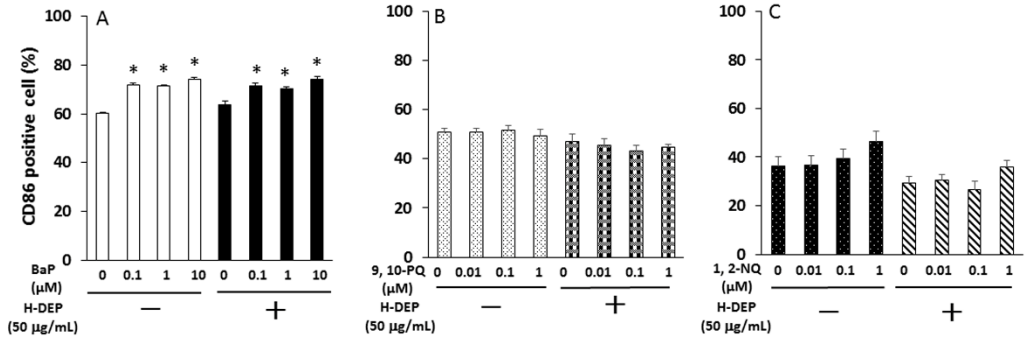


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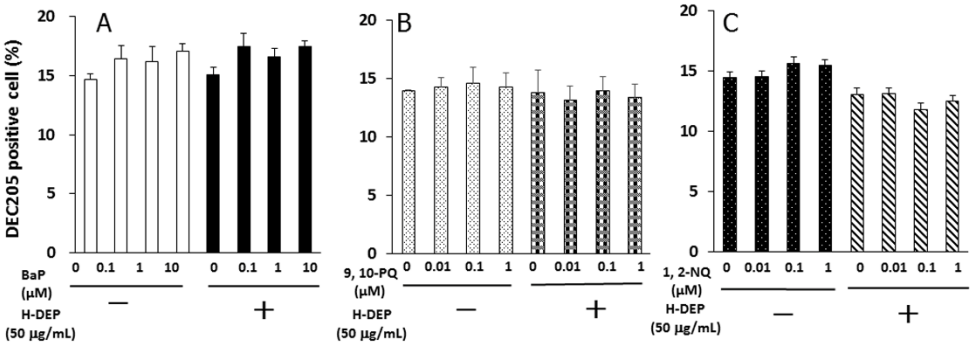
548 Fig.7

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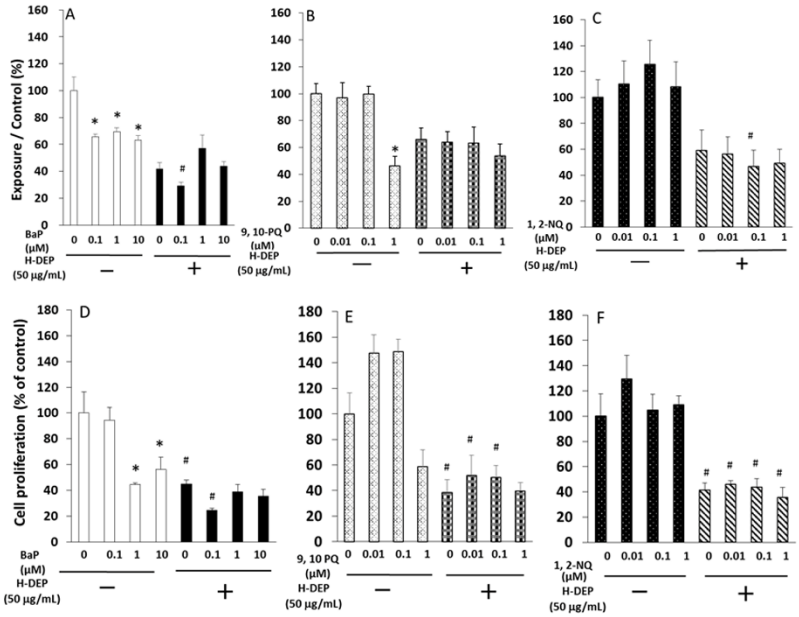


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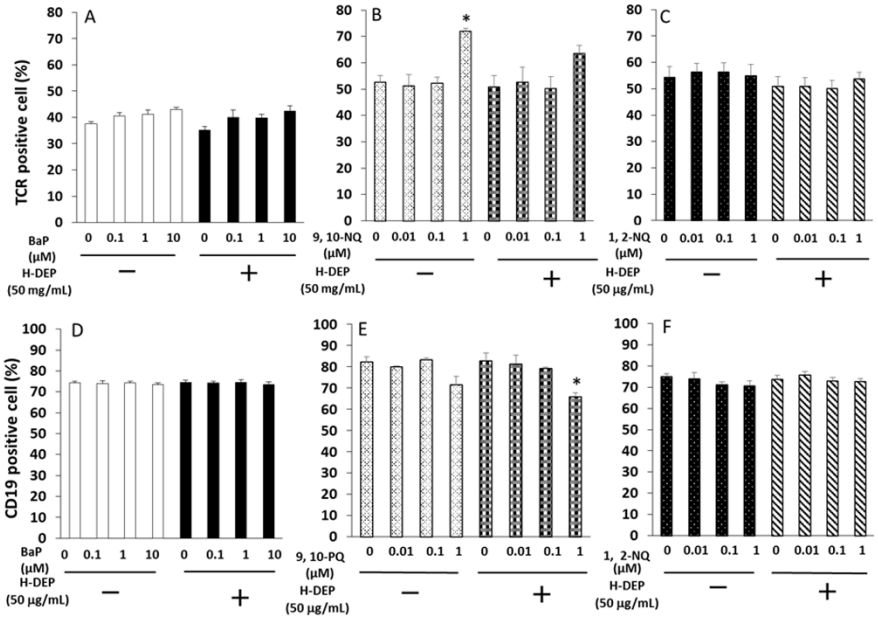
553 Fig.9

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